

RESEARCH PAPER

Endogenous cannabinoids induce fever through the activation of CB₁ receptorsD Fraga¹, CIS Zanoni², GA Rae³, CA Parada⁴ and GEP Souza¹¹Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, University of São Paulo, Ribeirão Preto, São Paulo, Brazil,²Laboratory of Pain, Faculty of Medicine, University of São Paulo, Ribeirão Preto, São Paulo, Brazil, ³Department of Pharmacology, Biological Sciences Center, Federal University of Santa Catarina, Florianópolis, Santa Catarina, Brazil, and⁴Biology Institute, State University of Campinas, UNICAMP, Campinas, São Paulo, Brazil

Background and purpose: The effects of centrally administered cannabinoids on body core temperature (T_c) and the contribution of endogenous cannabinoids to thermoregulation and fever induced by lipopolysaccharide (LPS) (Sigma Chem. Co., St. Louis, MO, USA) were investigated.

Experimental approach: Drug-induced changes in T_c of male Wistar rats were recorded over 6 h using a thermistor probe (Yellow Springs Instruments 402, Dayton, OH, USA) inserted into the rectum.

Key results: Injection of anandamide [(arachidonylethanolamide (AEA); Tocris, Ellisville, MO, USA), 0.01–1 µg i.c.v. or 0.1–100 ng intra-hypothalamic (i.h.), induced graded increases in T_c (peaks 1.5 and 1.6°C at 4 h after 1 µg i.c.v. or 10 ng i.h.). The effect of AEA (1 µg, i.c.v.) was preceded by decreases in tail skin temperature and heat loss index (values at 1.5 h: vehicle 0.62, AEA 0.48). Bell-shaped curves were obtained for the increase in T_c induced by the fatty acid amide hydrolase inhibitor [3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate (Cayman Chemical Co., Ann Arbor, MI, USA) (0.001–1 ng i.c.v.; peak 1.9°C at 5 h after 0.1 ng) and arachidonyl-2-chloroethylamide (ACEA; Tocris) (selective CB₁ agonist; 0.001–1 µg i.c.v.; peak 1.4°C 5 h after 0.01 µg), but (*R,S*)-(+)-(2-Iodo-5-nitrobenzoyl)-[1-(1-methyl-piperidin-2-ylmethyl)-1*H*-indole-3-yl] methanone (Tocris) (selective CB₂ agonist) had no effect on T_c. AEA-induced fever was unaffected by i.c.v. pretreatment with 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indole-3-yl(4-methoxyphenyl) methanone (Tocris) (selective CB₂ antagonist), but reduced by i.c.v. pretreatment with N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251; Tocris) (selective CB₁ antagonist). AM251 also reduced the fever induced by ACEA or LPS.

Conclusions and implications: The endogenous cannabinoid AEA induces an integrated febrile response through activation of CB₁ receptors. Endocannabinoids participate in the development of the febrile response to LPS constituting a target for antipyretic therapy.

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Keywords: anandamide; ACEA; AM251; AM630; AM1241; URB597; capsazepine; fever; LPS

Abbreviations: ACEA, arachidonyl-2-chloroethylamide; AEA (anandamide), arachidonylethanolamide; AH/POA, anterior hypothalamic/preoptic area; AM1241, (*R,S*)-(+)-(2-Iodo-5-nitrobenzoyl)-[1-(1-methyl-piperidin-2-ylmethyl)-1*H*-indole-3-yl] methanone; AM251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AM630, 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indole-3-yl(4-methoxyphenyl) methanone; CPZ, capsazepine; CRF, corticotropin releasing factor; Δ⁹-THC, Δ⁹-tetrahydrocannabinol; ΔT_c, changes from the mean basal core temperature value; FAAH, fatty acid amide hydrolase; HLI, heat loss index; HU-210, [(BaR)-trans-3-(1,1-dimethylheptyl)-6α,7,10,10α-tetrahydro-1-hydroxy-6,5-dimethyl-βH-dibenzo-[b,d]pyran-9-methanol]; i.h., intra-hypothalamic; LPS, lipopolysaccharide; SR141716A, [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride]; Ta, ambient temperature; T_c, core temperature; TRPV1, transient receptor potential vanilloid 1; Ts, tail skin temperature; URB597, [3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate; WIN-55212-2, [(4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-naphthalenyl-carbonyl)-6*H*-pyrrolo[3,2,1-*ij*]quinolin-6-one)-pyrrolo[3,2,1-*ij*]quinolin-6-one]

Introduction

Fever is a multi-mediated response that integrates the complex physiological defence strategy of the host that follows the recognition of invading microbial or non-microbial organisms as foreign by immune cells. Recognition

of the invader activates the production and release of a diverse array of peptidic and non-peptidic mediators, which includes many cytokines, corticotropin releasing factor (CRF), endothelins (Dinarello, 1984; Dinarello *et al.*, 1986; Helle *et al.*, 1988; Rothwell, 1990; Fabricio *et al.*, 1998), endogenous opioids (Fraga *et al.*, 2008) and prostaglandins (Dinarello *et al.*, 1991; Fraga *et al.*, 2008). Such mediators activate mechanisms that ultimately increase the thermoregulatory set-point located in the hypothalamus to promote the febrile response (Zeisberger, 1999; Roth and Souza, 2001 for review).

The recreational, medicinal and ritualistic uses of *Cannabis sativa* date back thousands of years, but it was only in the 1960s that the plant's main active constituent, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), was isolated and structurally characterized (Mechoulam and Gaoni, 1965a,b). Since then, it has been found that Δ^9 -THC largely mimics the effects of the lipidic endocannabinoid anandamide [arachidonylethanolamide (AEA)] (Devane *et al.*, 1992) and 2-arachidonoylglycerol (Mechoulam *et al.*, 1995), which promote widespread effects through activation of two specific G protein-coupled cannabinoid receptors, namely CB₁ (Matsuda *et al.*, 1990) and CB₂ receptors (Munro *et al.*, 1993) respectively. These receptors can signal inhibitory effects on adenylyl cyclase and calcium channel activities, and activate mitogen-activated protein kinases and potassium channels (Mackie, 2008). CB₁ receptors are heterogeneously distributed within the central nervous system (CNS) and can account for several of the characteristic pharmacological properties of cannabinoid receptor agonists (Pertwee, 2005; 2008 for review). CB₂ receptors are present mainly in the periphery, have been detected in cells of the immune system and are responsible for the immunoregulatory effects of cannabinoids (Pertwee, 1997; Howlett, 2002). In addition, AEA can also interact with, and stimulate, capsaicin-sensitive transient potential receptor vanilloid 1 (TRPV1) receptors (Rice *et al.*, 2002; Pertwee, 2005). Inactivation of endocannabinoids is dependent on two mechanisms, reuptake and degradation (Beltramo *et al.*, 1997; Hillard and Campbell, 1997; Hillard *et al.*, 1997). The fatty acid amide hydrolase (FAAH) is the main enzyme responsible for the degradation of AEA to arachidonic acid and ethanolamide (Cravatt *et al.*, 1996; Goparaju *et al.*, 1999a,b).

It has long been recognized that rodents treated with cannabinoids display four characteristic symptoms: analgesia, catalepsy, hypoactivity and hypothermia (Lomax and Campbell, 1971; Pertwee, 2005 for review). Despite the prominence of hypothermia [i.e. a reduction in body core temperature (T_c)], this symptom is only observed in animals administered high doses of Δ^9 -THC. In fact, at lower doses, Δ^9 -THC increases rather than decreases T_c (Sofia, 1972; Fennessy and Taylor, 1977). As to the effects of endocannabinoids on T_c, Crawley *et al.* (1993) and Chaperon and Thiebot (1999) have shown that systemic administration of AEA induces a dose-dependent reduction in T_c. The effects of central administration of natural and synthetic cannabinoids on T_c have also been explored. In this regard, dose-dependent hypothermia has been observed following microinjections of Δ^9 -THC into the anterior hypothalamic/preoptic area (AH/POA) in mice (Fitton and Pertwee, 1982), or of the synthetic cannabinoid agonists [(BaR)-trans-3-(1,1-dimethylheptyl)-6 α ,7,10,10 α -

tetrahydro-1-hydroxy-6,5-dimethyl- β H-dibenzo-[b,d]pyran-9-methanol] (HU-210) (Ovadia *et al.*, 1995) or [(4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-naphthalenyl-carbonyl)-6H-pyrrolo[3,2,1*ij*]quinolin-6-one)-pyrrolo[3,2,1*ij*]quinolin-6-one] (WIN-55212-2) in rats (Rawls *et al.*, 2002). The latter study suggested that CB₁ receptors within the AH/POA are responsible for the signalling of cannabinoid-induced hypothermia, as the effect of WIN-55212-2 was dose-dependently antagonized by intramuscular treatment with [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride] (SR141716A), a selective CB₁ receptor antagonist.

However, the effects of centrally administered endogenous cannabinoids on T_c responses and the role of CB₁ and CB₂ receptors in bringing about their putative effects are currently unknown. The present study was designed to examine this issue, and also the possible involvement of central endocannabinoids and their receptors in thermoregulation and the febrile response to LPS.

Methods

Animals

Male Wistar rats weighing 180–200 g, housed 4–5 per cage at 24 ± 1°C under a 12:12 h light–dark cycle (lights on at 7 h, 0 min) with free access to food chow and tap water were used in this study. All experiments were approved by the institution's Ethics Committee for Research On Laboratory Animal Use and are in accordance with the guidelines set by the US National Institute of Health and Brazilian legislation.

Intracerebral cannula implantation and microinjections

Under anaesthesia with sodium pentobarbitone (Sigma Chem. Co., St. Louis, MO, USA) (40 mg·kg⁻¹, i.p.), a permanent 22-gauge stainless-steel guide cannula (0.8 mm outer diameter, 10 mm long) was stereotaxically implanted into the right lateral ventricle at coordinates: 1.6 mm lateral to the midline, 1.5 mm posterior to bregma and 2.5 mm under the brain surface, and the incisor bar was lowered 2.5 mm below the horizontal zero (Paxinos *et al.*, 1985). In other rats, for intra-hypothalamic (i.h.) injection, a 24-gauge stainless-steel guide cannula (0.55 mm outer diameter, 15 mm long) was stereotaxically implanted unilaterally into the AH/POA. Its stereotaxic coordinates were: 0.6 mm lateral to the midline, 7.7 mm anterior to the interaural line and 6.5 mm under the brain surface, and the incisor bar was lowered 3.0 mm below the horizontal zero (Paxinos *et al.*, 1985). Cannulae were fixed to the skull with jeweller's screws embedded in dental acrylic cement. All procedures were conducted under aseptic conditions, and the rats were treated with oxytetracycline hydrochloride (400 mg·kg⁻¹, i.m.) and allowed to recover for 1 week prior to experimental use.

Pyrogenic stimuli were all injected between 10:00 and 11:00 A.M. Microinjections into the lateral ventricle (i.c.v.) and into the AH/POA (i.h.) were made aseptically using a 30-gauge needle connected by polyethylene (PE 10) tubing to a 25 or 10 μ L Hamilton syringe (Sigma Aldrich, St. Louis, MO, USA)

respectively. The needle protruded 2 mm beyond the cannula tip, and a 2 μ L (i.c.v.) or 200 nL (i.h.) volume was injected slowly (over 1 min) with Hamilton syringe. For i.h. injections the syringe was coupled to a microinfusion pump (model KDS101, KD Scientific, EUA, Holliston, MA, USA). After the injection, the needle remained in place for 30 s before it was withdrawn to prevent backflow of the injection fluid through the cannula.

After the experiments, rats received a microinjection of Evan's blue (2.5%) either into the lateral ventricle (5 μ L) or into the POA (500 nL), as appropriate, followed by an overdose of pentobarbitone, and were perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde. Each brain was removed, stored in the same fixative for 6 h, kept in 30% sucrose overnight and cut in 40 μ m coronal sections on freezing microtome to assess, under light microscopy, the position of the cannulae and respective sites of perfusion. Animals showing cannula misplacement or blockage upon injection, or abnormal body weight gain patterns during the post-implantation period were excluded from the study.

Temperature measurements

Tc was measured by inserting a thermistor probe (Yellow Springs Instruments 402, Dayton, OH, USA) 4 cm into the rectum for 1 min, at 30 min intervals, for up to 6 h. For each measurement, the animal was picked up gently and held by the experimenter during temperature measurements, without removing the animal from its home cage. This procedure was performed at least twice on the day before the experiment to minimize temperature changes secondary to handling. On the day of the experiment, the basal Tc of each animal was estimated four times, at 30 min intervals, before any injection. Only animals displaying a mean basal Tc between 36.8 and 37.2°C were selected for the study. The experiments were conducted during the light cycle in a temperature-controlled room of $28 \pm 1^\circ\text{C}$, the thermoneutral zone for rats (Gordon, 1990; Romanovsky *et al.*, 2002). In some experiments, tail skin temperature (Ts) was measured, without removing the rats from their home cages, by attaching a thermistor probe to the lateral surface of the most distal third of the tail for 1 min, at 30 min intervals, up to 6 h. The thermistor probe was fixed and isolated from the changes of ambient temperature (Ta) by a thermal isolating tape laying over a piece of micropore to avoid tail skin irritation. On the day of the experiment, the basal Ts of each animal was determined four times, at 30 min intervals, before any injection. Only animals displaying a basal Ts between 32.0 and 33.0°C were selected for the study.

To indirectly estimate the changes in the peripheral vasomotor tone, the heat loss index (HLI) was calculated to eliminate direct influences of both Ta and Tc on Ts, according to the formula:

$$\text{HLI} = (\text{Ts} - \text{Ta}) / (\text{Tc} - \text{Ta})$$

The value of HLI can vary from 0 (maximal vasoconstriction) to 1 (maximal vasodilatation) (Romanovsky *et al.*, 2002).

Experimental design

In the first set of experiments, we simultaneously evaluated the effects of i.c.v. injections of AEA on Tc and Ts, to enable calculation of the HLI. The effect of i.h. injections of AEA was also evaluated on Tc only.

The next set of experiments investigated the effects of i.c.v. injections of [3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate (URB597; Cayman Chemical Co., Ann Arbor, MI, USA) (an inhibitor of FAAH), arachidonyl-2-chloroethylamide (ACEA; Tocris) (a selective CB₁ receptor agonist) and (R,S)-(+)-(2-Iodo-5-nitrobenzoyl)-[1-(1-methylpiperidin-2-ylmethyl)-1H-indole-3-yl] methanone (AM1241; Tocris) (a selective CB₂ receptor agonist) on Tc.

Another set of experiments was designed to confirm the identities of the receptors implicated in febrile responses induced by AEA and ACEA. The animals were pretreated (i.c.v.) with N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251; Tocris) (a selective CB₁ receptor antagonist), 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indole-3-yl](4-methoxyphenyl) methanone (AM630; Tocris) (a selective CB₂ receptor antagonist) or capsazepine (CPZ; Sigma Chem. Co.) (an antagonist of TRPV1 – or vanilloid – receptors) 30 min before i.c.v. administration of AEA or ACEA.

A final experiment was designed to investigate the contribution of central cannabinoid CB₁ receptor-operated mechanisms to the febrile response induced by LPS. The animals were pretreated (i.c.v.) with AM251 30 min before the i.p. administration of LPS.

Statistical analysis

For data analysis of changes in Tc and Ts, the baseline temperatures before any treatment were calculated for each animal by averaging the last three temperature measurements before any injection, and all subsequent temperatures were expressed as changes from the mean basal core temperature value (ΔTc). HLI was calculated as described earlier. All data are reported as means \pm SEM and were analysed for statistical significance by two-way analysis of variance followed by Bonferroni's test. The data were analysed using Prism Software (Graph-Pad, San Diego, CA, USA). Significance was set at $P < 0.05$.

Drugs and materials

AEA (anandamide), ACEA, AM1241, AM251 and AM630, all purchased from Tocris (Ellisville, MO, USA), and URB597 (Cayman Chemical Co.) were all diluted in saline containing propylene glycol (10%) and Tween 80 (1%). CPZ (Sigma Chem. Co., St. Louis, MO, USA) was diluted in saline containing ethanol (20%) and cremophor (20%). Lipopolysaccharide (LPS, *Escherichia coli* 0111:B4, Sigma Chem. Co.) was diluted in saline. Sodium pentobarbitone (Sigma Chem. Co.); microinfusion pump (KD Scientific); thermistor probe (Yellow Springs Instruments, model 402).

Results

Central effects of AEA on Tc

Figure 1A shows that the i.c.v. administration of AEA at doses of 0.01, 0.1, 1 and 10 μ g (injected in 2 μ L) induced long-

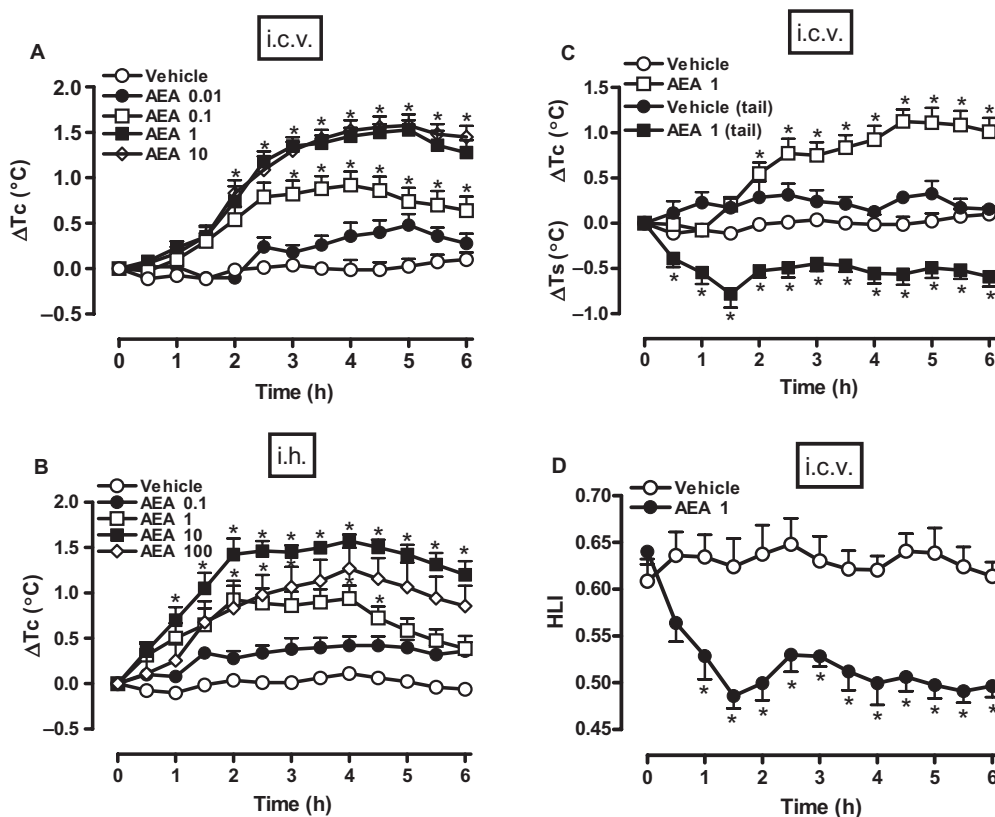


Figure 1 Anandamide [arachidonylethanolamide (AEA), i.c.v. and i.h.] increased core temperature (Tc) and reduced tail skin temperature (Ts) and heat loss index (HLI). AEA (or vehicle) was injected either by the i.c.v. route (in 2 µL), at 0.01, 0.1, 1 or 10 µg (A), or at 1 µg (C) or by the intra-hypothalamic (i.h.) route (in 0.2 µL), at 0.1, 1, 10 or 100 ng (B). Values of HLI in (D) were calculated from results depicted in (C), as described in Methods. Each value is the mean ± SEM of the change in Tc [changes from the mean basal core temperature value (ΔT_c), in °C] observed in 6–10 rats in (A) and (B), or of the change in Tc or Ts (ΔT_c or ΔT_s , respectively, in °C) (C) and HLI (D) observed in 7–10 rats. * $P < 0.05$ compared to vehicle-treated group.

lasting and dose-dependent increases in Tc that started at 2 h after administration and peaked around 5 h. As the increase in Tc induced by 1 µg of AEA was clearly maximal, this dose was selected to assess the simultaneous effects of the endocannabinoid on Tc and Ts. The experiments showed that the increase in Tc induced by i.c.v. AEA was clearly preceded (and accompanied) by significant and sustained reductions in Ts (Figure 1C), which resulted in decreases in calculated HLI (Figure 1D). Thus, AEA evoked an integrated thermoregulatory response comprising increases in Tc concomitant with cutaneous vasoconstriction. Importantly, i.h. injections of AEA (0.1, 1, 10 and 100 ng, in 0.2 µL) into the AH/POA promoted dose-dependent increases in Tc, with a maximal effect at 10 ng (Figure 1B). The onset (1 h) of the response to i.h. AEA and the time elapsed to reach a plateau (2–3 h) were shorter than those observed following i.c.v. injection.

Effects of URB597 (FAAH inhibitor), ACEA (CB₁ agonist) and AM1241 (CB₂ agonist) on Tc

The i.c.v. administration of URB597 or ACEA (both at 0.001–1 µg, in 2 µL) induced bell-shaped increases in Tc. The dose of 0.1 µg URB597 induced the greatest increase in Tc, which started 1.5 h after administration and peaked at 5 h (Figure 2A). The most effective dose of ACEA was 0.01 µg,

which raised Tc significantly at 3 h after injection to attain a peak at 5 h (Figure 2B). However, the i.c.v. administration of the selective CB₂ receptor agonist AM1241 (0.01–1 µg, in 2 µL) did not affect Tc values significantly (Figure 2C).

Effect of AM251 (CB₁ antagonist), AM630 (CB₂ antagonist) and CPZ (TRPV1 antagonist) on fever induced by AEA and ACEA

Prior i.c.v. treatment with AM251 (1, 5 and 10 µg) inhibited the development of the febrile response to AEA (1 µg, i.c.v.), being most effective at the dose of 5 µg (Figure 3A). At the latter dose, AM251 also attenuated substantially the fever induced by ACEA (0.01 µg, i.c.v.; Figure 3B). However, neither AM630 (10 µg, i.c.v.) nor CPZ (1, 3 and 10 µg, i.c.v.) influenced the fever induced by AEA (Figure 3C,D respectively). Importantly, Figure 3 also shows that i.c.v. injections of AM251, AM630 or CPZ alone (each at the highest doses) failed to alter the Tc of control animals.

Effect of AM251 (CB₁ antagonist) on fever induced by LPS

Systemic administration of LPS (50 µg·kg⁻¹, i.p.) induced an increase in Tc that started 1 h after injection, peaked at 2.5 h and remained at this level until 6 h. Pretreatment of the animals with the selective CB₁ receptor antagonist AM251

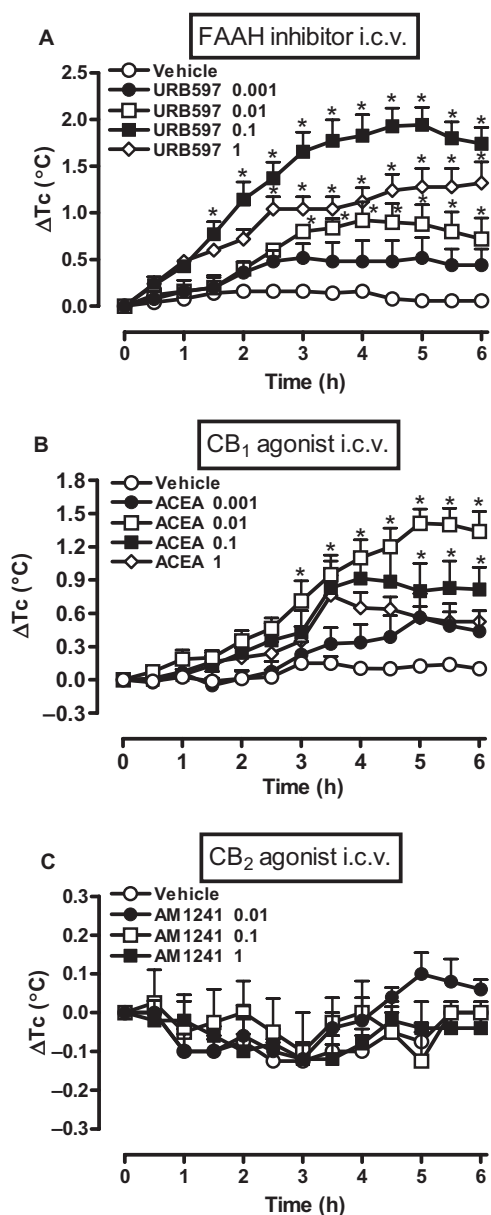


Figure 2 A fatty acid amide hydrolase (FAAH) inhibitor (URB597) and a CB₁ receptor agonist [arachidonyl-2-chloroethylamide (ACEA)] increased core temperature (T_c), but a CB₂ receptor agonist (AM1241) did not. Rats received i.c.v. injections of (A) URB597 (0.001, 0.01, 0.1 or 1 ng), (B) ACEA (0.001, 0.01, 0.1 or 1 μg) or (C) AM1241 (0.01, 0.1 or 1 μg), or an equal volume of the corresponding vehicle (2 μL). Each value is the mean ± SEM of the change in T_c [changes from the mean basal core temperature value (ΔT_c), in °C] observed in 6–9 rats. **P* < 0.05 compared to vehicle-treated group. AM1241, (*R,S*)-(+)-(2-Iodo-5-nitrobenzoyl)-[1-(1-methylpiperidin-2-ylmethyl)-1*H*-indole-3-yl] methanone; URB597, [3-(3-carbamoylphenyl)phenyl] *N*-cyclohexylcarbamate.

(5 μg, i.c.v.) promoted a marked and long-lasting decrease in the fever induced by LPS, from the 1.5 h time point onwards (Figure 4). It is noteworthy that, besides inhibiting the magnitude of LPS-induced fever by about 50–70%, AM251 also delayed the onset of the response to LPS and the time to its peak effect. A higher dose of AM251 (10 μg) did not inhibit the response to LPS to a greater extent than 5 μg (results not shown).

Discussion

The present study shows that central administration of the endocannabinoid AEA promotes dose-dependent increases in T_c in rats, which appears to integrate a genuine febrile response. The effect of AEA on T_c was mimicked by i.c.v. injection of a selective CB₁ receptor agonist and prevented by treatment with a CB₁ receptor antagonist, whereas ligands selective for CB₂ receptors were inactive. Moreover, as blockade of AEA metabolism, by use of an inhibitor of FAAH, increased T_c, and LPS-induced fever was attenuated by blockade of central CB₁ receptors, central endocannabinoids appear to play a relevant physiological role in thermoregulation.

As cited in the Introduction, several studies have reported that systemic, i.c.v. or i.h. treatment with exogenous cannabinoids induces robust hypothermic responses in rodents (Holtzman *et al.*, 1969; Lomax and Campbell, 1971; Taylor and Fennessy, 1977; Rawls *et al.*, 2002), which are likely to be subsequent to stimulation of CB₁ receptors (Rawls *et al.*, 2002). Likewise, systemic (i.p.) AEA administration in rats was reported to cause solely hypothermic responses (Crawley *et al.*, 1993; Chaperon and Thiebot, 1999), which are blocked by i.p. treatment with the CB₁ receptor antagonist SR141716A (Costa *et al.*, 1999). Conversely, i.p. and p.o. administration of Δ¹-THC (Sofia, 1972) or i.v. Δ⁹-THC injection (Taylor and Fennessy, 1977) was found to cause dual effects in rats, that is, increases in T_c at lower doses, but profound hypothermia following higher doses. The current study, which appears to be the first to assess T_c responses to a centrally administered endocannabinoid, detected clear-cut sustained and dose-related increases in T_c following i.c.v. injection of AEA. Although CB₂ receptors are generally absent from the majority of brain cells, CB₁ receptors are widely, but heterogeneously, expressed throughout the CNS (Matsuda *et al.*, 1990; Munro *et al.*, 1993; Pertwee, 2005). Importantly, the hypothalamus is particularly enriched with CB₁ receptors (Tsou *et al.*, 1998), especially in the lateral hypothalamic area and preoptic anterior nucleus (Moldrich and Wenger, 2000), the latter being critically implicated in thermoregulation (Boulant, 2000). In agreement with this evidence, we observed that AEA was about 10-fold more potent in promoting increases in T_c when microinjected directly into the AH/POA than when the i.c.v. route of administration was employed.

By measuring T_c and T_s concomitantly, we also determined whether the increase in T_c induced by i.c.v. AEA constituted an integrated thermoregulatory response (i.e. fever) or was merely the result of hyperthermia. Indeed, the long-lasting increase in T_c caused by AEA (1 μg, i.c.v.) was preceded and accompanied by a reduction in T_s, which led to a sustained reduction of the calculated HLI, a reliable means of discriminating fever from hyperthermia (Romanovsky *et al.*, 2002). Therefore, AEA-treated rats clearly developed an integrated response comprising an increase in T_c associated with a significant decrease in heat dissipation (i.e. reduction in T_s) due to tail skin vasoconstriction, as is characteristic of the febrile response phenomenon in these species (Romanovsky *et al.*, 2002).

Even though AEA displays higher affinity for the CB₁ receptors, it can also interact with the CB₂ and TRPV1

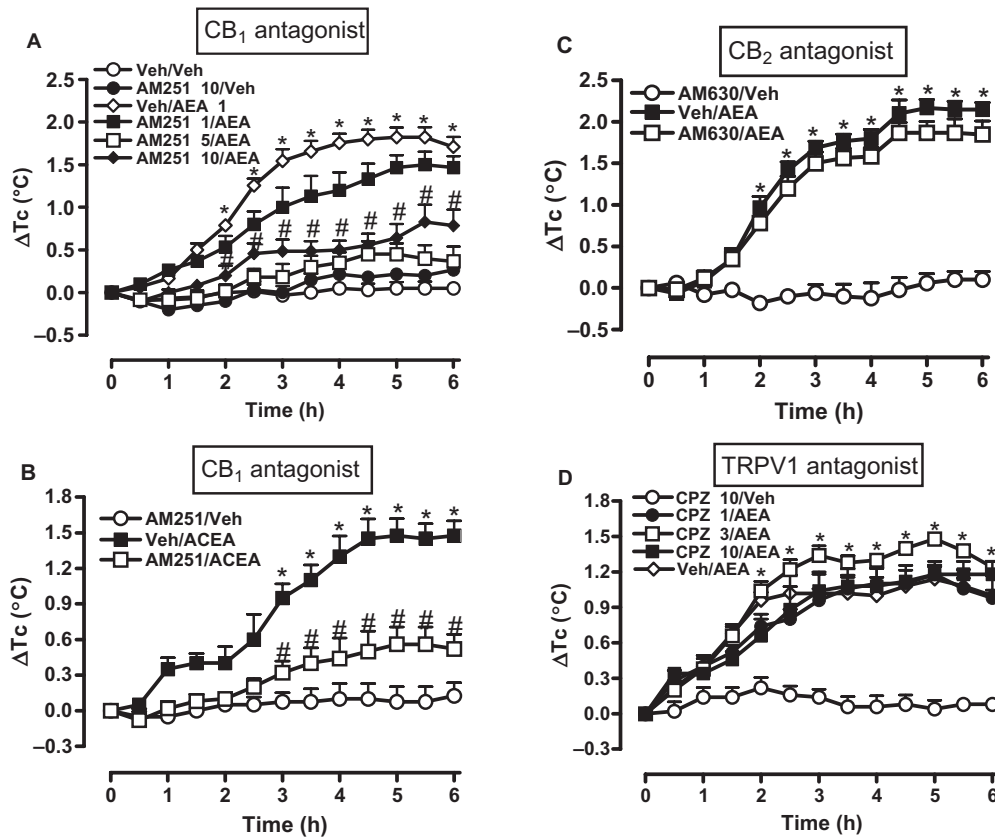


Figure 3 A selective antagonist of CB₁ receptors (AM251), but not of CB₂ receptors (AM630) or TRPV1 receptors [capsazepine (CPZ)], attenuated fever induced by arachidonylethanolamide (AEA) and another CB₁ receptor agonist [arachidonyl-2-chloroethylamide (ACEA)]. Rats received an i.c.v. injection of (A) AM251 at 1, 5 or 10 μg or (B) at 5 μg, (C) AM630 at 10 μg, (D) CPZ at 1, 3 or 10 μg, or an equal volume of corresponding vehicles (Veh, 2 μL) 30 min before i.c.v. injection of (A,C,D) AEA (1 μg), (B) ACEA (0.01 μg) or vehicle alone (2 μL). Each value is the mean ± SEM of the change in Tc [changes from the mean basal core temperature value (ΔTc), in °C] observed in 6–9 rats. **P* < 0.05 compared to AM251/Veh (A,B), AM630/Veh (C) or CPZ/Veh (D) group. #*P* < 0.05 compared to Veh/AEA (A,C,D) or Veh/ACEA (B) group. AM251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; AM630, 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indole-3-yl(4-methoxyphenyl) methanone; TRPV1, transient receptor potential vanilloid 1.

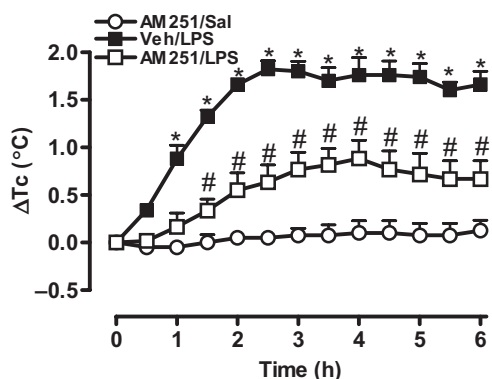


Figure 4 The selective CB₁ receptor antagonist AM251 reduced the febrile response induced by lipopolysaccharide (LPS). Rats received an i.c.v. injection of AM251 (5 μg) or equal volume of the vehicle (Veh, 2 μL) 30 min before i.p. injection of LPS (50 μg·kg⁻¹) or saline (Sal) (1 mL·kg⁻¹). Each value is the mean ± SEM of the change in Tc [changes from the mean basal core temperature value (ΔTc), in °C] observed in 6–7 rats. **P* < 0.05, #*P* < 0.05 when compared to AM251/Sal or Veh/LPS group respectively. AM251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide.

(vanilloid) receptors (Matsuda *et al.*, 1990; Munro *et al.*, 1993; Rice *et al.*, 2002; Pertwee, 2005). We thus attempted to carefully evaluate which of these targets was relevant for the development of AEA-induced fever. Our findings demonstrate that the fever induced by i.c.v. AEA was sensitive to dose-dependent inhibition by prior treatment with the CB₁ receptor antagonist AM251, but not the CB₂ receptor antagonist AM630. Moreover, i.c.v. injections of the CB₁ receptor agonist ACEA caused graded increases in Tc over a broad dose range, whereas similar treatment with the CB₂ receptor agonist AM1241 (up to 1 μg) did not change Tc. Finally, the development of AEA-induced fever was not modified by prior i.c.v. administration of CPZ, a TRPV1 receptor antagonist (Valenzano and Sun, 2004). Therefore, the evidence collected points to a pivotal role of CB₁ receptors in the signalling mechanisms that promote the AEA-induced fever response.

It is perhaps important to comment that i.c.v. ACEA yielded a bell-shaped dose-response curve for its Tc increasing effect, whereas AEA did not. This difference could be ascribed to: (i) the limited range of AEA doses chosen for the curve impeded the detection of the curve's downward deflection portion; (ii) at higher doses, ACEA (unlike AEA) may lose its selectivity

towards CB₁ receptors and act on additional AEA-insensitive targets; and/or (3) i.c.v. injection of high doses of CB₁ receptor agonists might stimulate hypothermic responses, as reported to occur in rats following systemic injection of endocannabinoids (Crawley *et al.*, 1993; Chaperon and Thiebot, 1999; Costa *et al.*, 1999). Further studies are needed to address each of these possibilities.

To investigate if central endocannabinoids participate in thermoregulation, we assessed the effects of an inhibitor of FAAH (URB597), the main enzyme responsible for AEA degradation (Cravatt *et al.*, 1996; Goparaju *et al.*, 1999a), on Tc, as well as the influence of the selective CB₁ receptor antagonist AM251 on fever induced by an exogenous pyrogen (LPS). Indeed, i.c.v. administration of URB597 (0.001–1 ng) caused graded increases in Tc, even though 1 ng induced a smaller effect than 0.1 ng. The reasons why this dose-response curve to URB597 displays a bell-shaped pattern remain to be clarified. Nonetheless, as inhibition of FAAH would be expected to increase endogenous AEA concentration in the synaptic cleft to enhance stimulation of CB₁ receptors, these data favour the hypothesis that endocannabinoids contribute to Tc regulation.

However, the febrile response to systemic (i.p.) LPS injection was reduced by prior i.c.v. injection of the CB₁ receptor antagonist AM251, at a dose that did not alter Tc in control rats *per se*. This constitutes strong functional evidence for the participation of the central endocannabinoid system in mediating, at least partially and via CB₁ receptor-operated mechanisms, the fever promoted by this exogenous pyrogen. A recent study (Benamar *et al.*, 2007) also reported that systemic administration of the selective CB₁ receptor antagonist SR141716A prevented the development of fever induced by LPS (50 µg·kg⁻¹, i.p.) in rats. Surprisingly, the same study found that i.p. injections of the CB₁ selective agonist WIN-55212-2 or Δ⁹-THC, at doses that did not cause hypothermia *per se*, also inhibited the febrile response to LPS, and that the antipyretic effect of WIN-55212-2 (which also reduced plasma interleukin-6 levels) was prevented by SR141716A. The authors suggested that their unexpected findings could perhaps be due to a partial agonistic activity of SR141716A at CB₁ receptors. The different findings, concerning Tc changes induced by systemic (Benamar *et al.*, 2007) and i.c.v. (present study) injections of CB₁ receptor agonists and antagonists, might indicate that central and peripheral endocannabinoids have distinct modulatory functions in the thermoregulatory changes induced by pyrogens.

In summary, the current study has shown that i.c.v. injection of a CB₁ (but not CB₂) receptor agonist causes sustained increases in Tc. This effect is associated with simultaneous peripheral vasoconstriction and a reduction in HLI, thus characterizing an integrated febrile response. Importantly, as central injection of an inhibitor of AEA catabolism raised Tc, and LPS-induced fever was sensitive to attenuation by central CB₁ receptor blockade, brain endocannabinoids seem to be implicated in pyrexia. We are presently engaged in studies to determine at what level(s) endocannabinoids are recruited to take part in the complex central signalling mechanisms triggered by LPS to provoke fever. Such studies could help establish the potential of central CB₁ receptors as a new relevant target for antipyretic therapy.

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Conflict of interest

None.

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